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Porcupine-mediated lipidation is required for Wnt recognition by Wls

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Abstract

Wnt proteins are members of a conserved family of secreted signaling ligands and play crucial roles during development and in tissue homeostasis. There is increasing evidence that aberrant Wnt production is an underlying cause of dysregulated Wnt signaling, however little is known about this process. One protein known to play a role in secretion is the transmembrane protein Wntless (Wls). However, the mechanism by which Wls promotes Wnt secretion is a riddle. It is not known which Wnt family members require Wls and what the structural requirements are that make some of them reliant on Wls for secretion. Here we present a systematic analysis of all known Drosophila Wnt family members with respect to their dependence on Wls function for secretion. We first show that the glycosylation status of Wg at conserved sites does not determine its dependence on Wls. Moreover, in apparent contrast to murine *wls*, Drosophila *wls* is not a target gene of canonical Wnt signaling. We then show that all Wnts, with the exception of WntD, require Wls for secretion. All Wnts, with the exception of WntD, also contain a conserved Serine residue (in Wg S239), which we show to be essential for their functional and physical interaction with Wls. Finally, all Wnts, with the exception of WntD, require the acyltransferase Porcupine for activity and for functionally interacting with Wls. Together, these findings indicate that Por-mediated lipidation of the S239-equivalent residue is essential for the interaction with, and secretion by, Wls.

Keywords:

Wntless; Porcupine; Wnt signalling; secretion; acylation

Introduction

Wnt proteins are a family of cysteine-rich, secreted glycoproteins that are encoded in all animal genomes. Wnt signaling regulates multiple diverse processes during animal development and controls tissue homeostasis in the adult. Dysregulation of the pathway is the cause for several hereditary diseases, and is also associated with various cancers, including intestinal cancer (Clevers 2006; MacDonald et al., 2009). The signaling events triggered by Wnts are relatively well understood; however the mechanisms underlying Wnt secretion are only poorly mapped out. Given the emerging relevance of Wnt production as an underlying cause of dysregulated Wnt signaling closing this gap is imperative.

Briefly, what is known about Wnt secretion: newly synthesized Wnts are lipid modified in the ER - a reaction that reportedly requires the acyltransferase Porcupine (Por) (Kadowaki et al., 1996). From the ER, Wnts are transported to the Golgi complex, where they encounter the transmembrane protein Wntless (Wls) (Banziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). Wls supports transport of Wnts from the Trans-Golgi Network (TGN) to the plasma membrane. From the plasma membrane Wls (and maybe some Wnt protein) is retrieved by clathrin-mediated endocytosis; Wls is then routed by the retromer complex into a retrograde trafficking pathway back to the TGN (Belenkaya et al., 2008; Franch-Marro et al., 2008; Pan et al., 2008; Port et al., 2008; Yang et al., 2008).

The mechanism by which Wls promotes Wnt secretion is enigmatic. It is not known why Wnts require Wls for secretion, nor which Wnts depend on Wls or what the structural requirements are that make some of them Wls independent. To date, one Wnt has been reported to be Wls-independent: WntD (also known as Wnt8) (Ching et al., 2008). It plays a role in dorsal-ventral patterning in the Drosophila embryo as well as during the innate immune response in adult animals (Gordon et al., 2005). Interestingly, WntD was found to be free of lipid-modifications (Ching et al., 2008). This was somewhat surprising as lipid modification was generally presumed to be a universal feature of Wnt proteins. Is lipid modification a pre-determinant for the requirement of Wls?

To address these questions and to try to elucidate if there are other features which make Wnts dependent on Wls for efficient secretion we set out to systematically characterize the interaction of all seven Drosophila Wnts with Wls. Our findings

suggest that the posttranslational modification of Wnts with lipid chains at a conserved Serine residue (Takada et al., 2006), corresponding to S239 in Wg, results in their recognition by Wls as cargo. Our results also point to Porcupine as being responsible for this modification in all the lipidated Drosophila Wnts. In contrast, neither the glycosylation status nor the type of signaling cascade activated by a Wnt is a determinant for the recognition by Wls.

Material and Methods

Fly stocks

Transgenics on landing sites ZH-22A for the *hsp70* promotor constructs or ZH-51D for the *UAS* promotor constructs:

Wg; Wg^{C93A}; Wg^{S239A}; $Wg^{C93A,S239A}$ ($Wg^{C,S}$); Wg^{N103A}; Wg^{N108A}; Wg^{N414A}; $Wg^{N103A,108A,414A}$ (Wg^{N3}); HAWg; $HAWg^{C93A}$; $HAWg^{S239A}$; $HAWg^{C93A,S239A}$; $HAWg^{N103A}$; $HAWg^{N108A}$; $HAWg^{N414A}$; $HAWg^{N103A,N108A,N414A}$; DWnt2; DWnt2-3xHA, $DWnt2^{S202A}$-3xHA; DWnt4; DWnt4-3xHA; $DWnt4^{S403A}$-3xHA; DWnt5; DWnt5-3xHA; $DWnt5^{S868A}$-3xHA ;DWnt6; DWnt6-3xHA; $DWnt6^{S283A}$-3xHA; DWntD; DWntD-3xHA; DWnt10; DWnt10-3xHA; $DWnt10^{S266A}$-3xHA; wls-lacZ; wls-wls(coding sequence); tubulinα1-porcupine-V5.

Other stocks:

yw ubiGFPnls hsp70-Flp tubGal80 FRT19; tubGal4
FRT19/FRT19
por^{2E} FRT19 / Fm7a
yw hsp70-Flp; sp/CyO; *wls*^{1} FRT80 / TM6B
yw hsp70-Flp ubiGFPnls FRT19; sp/CyO
yw hsp70-Flp ;; *dpp*-Gal4 / TM6B
yw; UAS-Flp/CyO; *dpp*-Gal4/TM6B
yw hsp70-Flp;;FRT80 ubiGFPnls $M(3)i^{55}$
yw hsp70-Flp; UAS-HA-NRTwg/CyO

Clonings, construct generations

DWnt coding sequences were PCR amplified and cloned into pUASTattB, phsp70attB and pAc5.1 expression vectors containing a C-termial 3xHA tag in frame using KpnI and BssHII restriction enzymes. Additionally we cloned the same coding regions without tag using KpnI/HindIII. The coding sequence of DWnt6 and DWnt10 had to be adjusted during the course of the study according to the recent annotation on Flybase and to fulfill criteria such as having a functional signal sequence (determined by using the SignalP 3.0 server (Jannick et al., 2004)). In the case of

DWnt10 we had to replace the most N-terminal sequence by the Wg signal peptide because the predicted N-terminus did not encode for one (see alignment in Figure S4).

The WntD$^{Wg235-246}$ construct was generated by exchanging 12 aminoacids around S239 in Wg from Wg to WntD.

Protein accession numbers used for the phylogenetic tree:

Drosophila sequences were obtained from Flybase: Wg (FBpp0079060), Wnt2 (FBpp0087596), Wnt4 (FBpp0088345), Wnt5 (FBpp0074394), Wnt6 (adapted from FBpp0292343), WntD (FBpp0082243) and Wnt10 (adapted from FBpp0292305).

C.Elegans sequences were obtained from Swiss-Prot: MOM2 (Q10459), Wnt1 (P34888), Wnt2 (P34889), LIN44 (Q27886) and EGL20 (A8WV58).

Zebrafish sequences were obtained from Swiss-Prot: Wnt7 (E7FBM9), Wnt11 (O73864), Wnt10A (P43446), Wnt5B (Q92050), Wnt2 (Q92048) or NCBI: Wnt16 (NP_001093516), Wnt7A (NP_001020711), Wnt4B (AF139536_1), Wnt2B (AAN62916), Wnt3 (NP_001108024) and Wnt5a (ABE96795).

Human sequences were obtained from Swiss-Prot: Wnt10B (O00744), Wnt4 (P56705), Wnt9B (O14905), Wnt3A (P56704), Wnt10A (Q9GZT5), Wnt7A (O00755), Wnt2B (Q93097), Wnt8B (Q93098), Wnt16 (Q9UBV4), Wnt5A (P41221), Wnt11 (O96014), Wnt9A (O14904), Wnt6 (Q9Y6F9), Wnt7B (P56706), Wnt2 (P09544), Wnt5B (Q9H1J7), Wnt3 (P56703), Wnt8A (Q9H1J5) and Wnt1 (P04628).

Secretion assay - *in vivo*

hsp70-Wg/Wnt-3xHA transgenes on ZH-22A were combined with the *wls*^{1} FRT80 chromosome and balanced over the compound Cyo-TM6B balancer. After crossing the resulting stocks to a *yw* hsp70-Flp;; FRT80 ubiGFPnls M(3)i^{55} strain, mitotic clones were induced by a 45 min. at 37°C heat-shock 3 days AEL and the non-TM6B larvae were subjected to a 2nd heat-shock for 90' at 37°C 1 hour before dissection to induce transgene expression.

Secretion assay - *in vitro*

S2R+ cells were transfected with the pAc5.1-Wg/Wnt-3xHA constructs using FuGeneHD (Promega). Equal numbers of transfected cells were then split and seeded in 96 well plates. The dsRNA for GFP as control and Wls were added to the serum free medium and cells were kept in SFM for 5 hours before the media was supplemented with serum. After 3 days the supernatant (SN) was exchanged and replaced with fresh 200µl Schneider's medium. After another 3 days the SN was collected and added to protein G-beads plus 0.2µg of rabbit anti-HA (805 Santa

Cruz) antibody for 2 hours at 4°C. The beads were washed twice in PBS the next day, the proteins were eluted from the beads in 20µl sample buffer and reducing agent (Invitrogen) for 5' at 95°C and subjected to western blot analysis. The antibody heavy chain bands are shown as an immunoprecipitation loading control. Protein levels in the cell lysate were immunblotted for α-HA and α-Tubulin as additional transfection controls.

Activity assay – *in vivo*

Each tagged and untagged UAS-Wnt construct was crossed into the *dpp*-Gal4 driver background. However, the constructs encoding Wg and or glycosylation mutant forms of Wg had toxic effects in the embryos and thus had to be established with a flp-out cassette between the UAS and the coding sequences; these constructs were crossed into a UAS-Flp;*dpp*Gal4 background. 3rd instar larvae were dissected and wing imaginal discs were stained for Senseless in combination with antibodies against Wg or the HA epitope.

Paracrine activity assays – *in vitro*

Kc cells were transfected with either the *wf*-Luc and *tubulinα1*-Renilla constructs or with the untagged pAc5.1-Wg/Wnt constructs using FuGeneHD (Promega). Cells were mixed the next day. Cells were lysed in passive lysis buffer (Promega) and the relative luciferase levels were measured on a Promega Glomax Multi Detection System after another 4 days. Cell lysate was immunoblotted for α-Wg and α-Tubulin as transfection controls.

Autocrine activity assay – *in vitro*

Kc cells were transfected with *wf*-Luc, *tubulinα1*-Renilla and the untagged pAc5.1-Wg constructs using FuGeneHD (Promega). Cells were lysed after 4 days in passive lysis buffer (Promega) and the relative luciferase levels were measured on a Promega Glomax Multi Detection System. Cell lysate was immunoblotted for α-Wg and α- Tubulin as transfection controls.

Immunoprecipitations (IP)

Co-IP's were performed on Kc cell lysate using ProteinG beads (Roche). Cell transfection was carried out using FuGeneHD transfection reagent (Promega). Cell lysis buffer: 50mM TrisHCl pH8, 140 mM NaCl, 1%TX100 with Proteinase Inhibitor Tablets from Roche. IP wash buffer for the Porcupine CoIP: 50mM TrisHCl pH7,

150mM NaCl. The Wls CoIP was performed using DSP (Thermo Scientific) crosslinking for 20 minutes at room temperature followed by 4 washing steps in wash buffer containing 65 mM TrisHCl pH7, 160 mM NaCl, 1%NP40. Proteins were eluted from the beads in 20µl sample buffer and reducing agent (Invitrogen) for 5' at 95°C and subjected to western blot analysis

PNGaseF assay

Kc cells were transfected with 3xHA tagged Wnt expression plasmids. After 3 days the supernatant was collected and subjected to immunoprecipitation to clean the samples from serum. Wnt protein bound to protein G beads (Roche) was digested with PNGaseF (New England Biolabs) following manufacturer protocols and subjected to western blot analysis.

Antibodies

Polyclonal rabbit anti-HA (1:500; 805 Santa Cruz); monoclonal mouse anti-HA (1:800; HA11 Covance); polyclonal rabbit anti-Wls (Port et al.,2008); polyclonal guinea pig anti-senseless (GP55 1:800; gift from H. Bellen, Baylor College of Medizine, Houston, TX); monoclonal mouse anti-Wg (4D4 1:1000 DSHB); monoclonal mouse anti-V5 (Invitrogen); mouse anti-Tubulin (Sigma); Alexa-Fluor 488 goat anti-mouse (1:500; Molecular Probes), Alexa-Fluor 594 goat anti-mouse (1:500; Molecular Probes), Alexa-Fluor 488 goat anti-rabbit (1:500; Molecular Probes), Alexa-Fluor 594 goat anti-rabbit (1:500; Molecular Probes), Alexa-Fuor 568 goat anti-guinea pig (1:500; Molecular Probes), goat anti-mouse Cy5 (1:500; Jackson Immunuresearch Laboratories), goat anti-rabbit-HRP (1:2000; Jackson Immunoresearch Laboratories), goat anti-mouse-HRP (1:2000; Jackson Immunoresearch Laboratories).

Immunostaining, microscopy and image analysis

Immunostainings were performed using standard protocols. All wing discs were fixed and imaged under the same conditions. Images were collected with a Zeiss LSM710 confocal microscope using 20x and 63x oil objectives. Images were processed with ImageJ. All images are single confocal sections close to the apical surface of the wing disc epithelium.

Quantification of the immunoblot bands was done using ImageJ gel analysis tools.

Results

Generating a tool-kit for systematically analyzing all Drosophila Wnts

Drosophila contains genes for seven Wnts: Wingless (Wg), Wnt2, Wnt4, Wnt5, Wnt6, Wnt10 and Wnt8/WntD. To be able to systematically test them we generated for all of them tagged (C-terminal 3xHA) and untagged expression constructs. As a reference we used the most recent annotation on Flybase (release date FB2010_07, September 3rd 2010). This was particularly relevant for DWnt6 and 10, which in earlier annotations had not included a functional signal sequence (Figure S1 and see Material and Methods for details). Alignments and ancestry analysis with all Wnts of different vertebrate and invertebrate species revealed that in the present situation all DWnts grouped well together with their corresponding orthologues in other species (Figure S2). Further, to circumvent potential position effects - and thus differences in expression levels – all constructs were integrated into the same attP landing site on the second chromosome using the phiC31 integrase (Bischof et al., 2007).

In cultured Drosophila cell lines, as well as in imaginal discs, we verified that all our tagged constructs were expressed and gave rise to secreted proteins (Figure S3A). With these tools in hand we could now test which of the Drosophila Wnts require Wls for secretion.

All Drosophila Wnts except WntD require Wls for secretion

To be able to monitor the secretion of Wnt proteins expressed at moderate levels we generated transgenics expressing the tagged Wnts under control of the *Hsp70* promoter. By varying the intensity of the heat-shock the expression levels can be fine-tuned and relatively low levels of expression can be achieved. Based on the accumulation of Wnt protein inside the cell and the reduced amount of extracellular protein we concluded that Wg, Wnt2, Wnt4, Wnt5, Wnt6 and Wnt10, but not WntD, were retained in cells lacking *wls* (Figure 1). The observed effect was not obvious for all Wnts, especially Wnt2. This might be explained by the relative background levels of the HA antibody. To get more evidence for the requirement of Wls by different Wnts we performed a secretion assay in cultured S2R+ cells. Wls function was depleted by RNAi mediated knock-down as in Port et al. (2011). Consistent with our *in vivo* results all Wnts except WntD needed Wls to be efficiently secreted from cultured S2R+ cells (Figure 1).

Golgi-relocalization of Wls reflects the requirement for Wls

We had previously observed, using an antibody against Wls, that in cells expressing Wg (endogenously or ectopically) Wls is relocalised to the Golgi apparatus (Port et al., 2008). This relocalization is seen as an apparent increase in Wls immunostaining (Figure 2). We wanted to test if the Golgi accumulation of Wls was elicited by all Wnts whose secretion is Wls-dependent. This was indeed the case and we found that in addition to Wg, also Wnt2, Wnt4, Wnt5, Wnt6 and Wnt10 induced a change in Wls localisation (Figure 2C-I). WntD, which does not require Wls for secretion, did not cause the Golgi-relocalization (Figure 2H). Thus the accumulation of Wls in the Golgi seems to reflect an underlying functional interaction between Wls and Wnts and can serve as a read-out for this functional interaction.

dWls is not a Wnt-target gene

We had postulated that the apparent increase in Wls immunostaining in the Golgi was due to posttranslational events (Port et al., 2008). However it had not been formally ruled out that in Drosophila increased *wls* expression contributes to the observed enhancement in Wls staining. Indeed it had recently been reported that murine *wls* was a direct target of canonical Wnt signalling (Fu et al., 2009). We were therefore curious if *dwls* expression was also regulated to any extent by canonical Wg pathway activity. To test this we generated a *wls* reporter construct for *in vivo* analysis: the first exon of the *wls* gene in a genomic rescue construct was replaced by the coding sequence of *lacZ* (Figure 2A). The construct contains all the essential regulatory elements because when a *wls* coding sequence was inserted at the same position the resulting transgene could rescue wls^{1} mutant animals. β-Galactosidase from the reporter construct was homogenously expressed in the wing imaginal disc and its levels were not enhanced in Wg-receiving cells. Therefore, in Drosophila, the apparently higher Wls levels in Wg producing cells are not due to a transcriptional response to Wg signalling (Figure 2B), but rather appear to be caused by enhanced recycling of Wls (Belenkaya et al., 2008, Franch-Marro et al., 2008, Pan et al., 2008, Port et al., 2008, Yang et al., 2008).

The ability to induce canonical Wnt signalling does not dictate Wls-dependence

Wnt-receptor interactions can elicit a variety of intracellular responses, the best known of which is the canonical pathway, which results in the modulation of target gene expression via the β-catenin/TCF transcriptional complexes. However, other intracellular messengers/signaling pathways can be activated by Wnt proteins, including calcium fluxes, Jnk and Src kinases, and the planar cell polarity pathway

(Segalen and Belaiche 2009). Since canonical signaling is the best understood we decided to assess the ability of the seven DWnts to activate the canonical Wnt signaling pathway a posteriori. Using Kc cells in a paracrine (co-culturing) assay we monitored the induction of canonical Wnt signaling with an established luciferase reporter for canonical Wg signaling. As expected, Wg strongly activated the reporter (Figure 3A). In addition, Wnt2 also could activate the canonical pathway; this was not unexpected, as it had previously been suggested to be able to act as a so-called canonical Wnt (Llimargas, M., Lawrence, P.A. 2001). The other Wnts had no discernable effect in this assay.

To test canonical pathway induction *in vivo* we expressed the UAS-Wnt constructs in the wing imaginal disc and monitored expression of *senseless (sens)*, which is a direct Wg target gene (Nolo et al., 2000). Consistent with previous reports Wg induced the expression of *sens* (Figure 3B). However, in contrast to the cell culture experiments, in this system Wnt2 had no effect. The other Wnts also did not activate *sens* expression (Figure 3). Taken together, our results show that both Wg and Wnt2 can induce canonical Wnt signaling, however the ability of Wnt2 to do so seems to be more restricted than that of Wg. This is consistent with previous work, which suggested that the Wnts may vary in their effectiveness in specific tissues, such as, for example, Wg and Wnt2 in the developing trachea (Llimargas, M., Lawrence, P.A. 2001). Importantly however, the results show that a dependence on Wls does not correlate with the ability to activate the canonical signaling pathway. This is further supported by our earlier observation that secretion of the non-canonical Wnt-5a in mice is dependent on Wls (Banziger et al., 2006). We next focused our attention on the post-translational modifications Wnts undergo: glycosylation and palmitoylation.

Glycosylation of Wnts at conserved sites is not required for their interaction with Wls

Wnts are predicted to be highly glycosylated proteins (Miller, 2001). We were therefore interested to test what impact glycosylation has on the secretion of Wnts and whether this modification is related to the Wls dependence. Wg has been shown to be normally glycosylated at N103 and N414 (Tanaka et al., 2002). Using the NetOGlyc 3.1 Server (Julenius et al., 2005) we determined that all Wnts, apart from WntD, are predicted to be glycosylated (Figure S4). We experimentally tested what Wnts are N-glycosylated by using the enzyme PNGaseF, which cleaves off N-linked glycans, and by examining the apparent molecular weight on western blots (Figure S3B). We found that, all Wnts, with the exception of WntD, are glycosylated.

To further investigate the link between glycosylation and Wls dependence we concentrated on Wg. The NetOGlyc 3.1 Server predicts Wg is potentially modified on N103, N108 and N414. The residues corresponding to N103 and N414 are also modified in Wnt3a (Komekado et al., 2007). We mutated all the Asn residues of Wg predicted by NetOGlyc 3.1 into Alanines. Like wild-type Wg, Wg variants lacking these glycosylation sites induced Wls to relocalise to the Golgi (Figure 4A-E). Confirming the Wls dependence, the variants all strongly accumulated in *wls* mutant cells in the wing imaginal disc (Figure 4A'-E'). These results indicate that glycosylation of Wg at these sites is not essential for the dependence on Wls for secretion.

As an aside we wanted to know what effect the glycosylation status of Wg at these sites has on its ability to activate the canonical signaling cascade. This we investigated in wing discs using *sens* induction as the readout and in paracrine (co-culturing) and autocrine activity assays in Kc cells (Figure S5). A Wg mutant that can not be glycosylated at these conserved sites is still able to induce *sens* expression in the wing disc and retained activity in tissue culture activity assays. From this we conclude that glycosylation at these conserved sites is dispensible for canonical Wg activity.

We note that Tanaka et al., 2002 found that Wg may also be glycosylated at N49. Consistent with this we observed that an overexpressed Wg mutant lacking N103, N108 and N414 in Kc cells can still be glycosylated (Figure S3C). If this occurs *in vivo* and has any functional relevance remains to be investigated. Nevertheless we generated a Wg N4 mutant (Figure S3C) and confirmed that this mutant is as well able to induce the canonical signaling pathway (Figure S5I) and requires Wls for secretion (Figure 4F). Our results show that the glycosylation at Asn conserved across Wnts does not impart Wls dependence.

<u>Por is essential for the recognition of Wnts by Wls.</u>

In addition to being glycosylated Wg is also lipid-modified. There are two palmitoylation sites known in Wg: Cysteine 93 with palmitate and Serine 239 with palmitoleic acid (Willert et al., 2003; Takada et al., 2006). It is likely that one or both of these modifications could impart Wls-dependence. Recently, Doubravska et al. (2011) reported that O-linked acylation of Serine of mWnt1 and mWnt3a is required for the subsequent S-palmitoylation of Cysteine. By extension, Wnt proteins that lack the crucial Serine residue would be expected not to be lipidated. Consistent with this, WntD which lacks the Serine residue (Figure 2J, Figure S4) is reportedly not lipid

modified (Ching et al., 2008). In the other Drosophila Wnts the Serine is present and by extension these Wnt are predicted to be lipid modified.

Since the acyltransferase responsible for these modifications is thought to be Porcupine (Hofmann 2000; Kadowaki et al., 1996) we chose as an initial approach to systematically analyze the effect of loss of *por* function on Wnt secretion and Wls localisation. As described previously in por^{2E} cells we observed that Wg fails to get secreted and strongly accumulates in these cells (Figure 5A, Van den Heuvel et al., 1989; Van den Heuvel et al., 1993). Interestingly we observed that in Wg-producing cells lacking *por* function Wls no longer relocalized to the Golgi (Figure 5A and B). In por^{+} control clones, like Wg, the other Wls-dependent Wnts - Wnt2, Wnt4, Wnt5, Wnt6 and Wnt10 - induced Wls to relocalise to the Golgi. This relocalization was completely blocked when these Wnts were expressed in por^{2E} clones (Figure 5B-G). These results suggest that whatever the action of Porcupine is it is important for the interaction of Wnts with Wls. This action is likely the acylation of Wnts.

S239 of Wg is essential for the recognition by Wls

As a next step we sought to test the two palmitoylation sites in Wg for their importance in Wls dependent secretion, and additionally for their contribution to Wg signaling activity. We generated transgenic animals carrying Wg^{C93A}, Wg^{S239A} and $Wg^{C93A,S239A}$ and analyzed the effect of these mutations in Wg on the Wls Golgi relocalization. To this end we expressed the variant Wg proteins in the wing disc with *dpp-Gal4* and monitored Wls protein distribution. Wg^{C93A} still induced Wls Golgi relocalization, whereas both Wg^{S239A} and $Wg^{C93A,S239A}$ failed to do so (Figure 6A-D). Like Wg, Wg^{C93A} accumulated in *wls* mutant cells. This is consistent with our earlier finding that the $Wnt3a^{C77A}$ mutant depends on Wls for secretion (Banziger et al., 2006). We also examined the secretion behavior of WgS239A and WgC93A,S239A. In agreement with the results of Franch-Marro et al. (2008) we found that Wg variants with the S239A change were secreted. Interestingly we did not observe enhanced accumulation of these Wg mutants in Wls clones (Figure 6A'-D'). Taken together, these results indicate that a mutant lacking one or both of the predicted acylations can still be secreted but whereas the C93A mutant still requires Wls, the S239A mutants appear not to. Thus palmitoylation of S239 in Wg correlates with its dependence on Wls for secretion.

The equivalent of $S239^{Wg}$ is essential for the recognition of all Wnts by Wls

The above results, together with the lack of conservation of the Serine in WntD, suggested that the Serine equivalent of Wg^{S239} could be essential for the recognition of a Wnt by Wls. To test this idea systematically we generated the corresponding S to A mutations in Wnt2, Wnt4, Wnt5, Wnt6 and Wnt10. These variants all lost their ability to induce Wls Golgi relocalization (Figure 7). The ability to bind Wls was also abrogated or clearly reduced as a consequence of the S to A mutation (Figure S6A).

The *in vivo* effects of mutating the palmitoylation site in Wnts are identical in outcome to expressing the wild-type Wnts in the absence of *por* function, indicating that Por acts on this conserved Serine. In an attempt to analyze this further we investigated whether Por could be co-immunoprecipitated with the DWnts. We found that Porcupine interacts with Wg, Wnt2, Wnt4, Wnt5, Wnt6 and Wnt10, but not WntD (Figure S6B). Supporting the assumption that the interaction was specific another secreted protein Eiger, a TNF superfamily ligand member, did not bind to Por. When we mutated the conserved Serine in the Wnts the interaction was strongly reduced (Figure S6B). The residual binding in the S-A Wnt variants indicates that while important the Serine is not the sole determinant of binding to Por. A WntD-Wg chimera ($WntD^{Wg235-246}$, Figure S6C) into which 12 amino acids flanking the Wg-S239 have been introduced into WntD is able to bind Por (Figure S6B). It was shown that Por binds to Wg at aminoacids 83-106 (Tanaka et al., 2002). However it seems that the sequence flanking the conserved serine is important for mediating the Wnt-Por interaction.

The fact that binding to Wls is not induced by transplanting the serine containing sequence suggests that the Wnt-Wls interaction and the Wnt-Por interaction are not mediated by the same motif. Our results do not reveal whether the subsitituted domain is sufficient to enable acylation by Por. But taken together our results show that acylation is a critical determinant for Wls-Wnt binding although the binding might be mediated elsewhere.

Finally, we asked whether the lipid modification of Wnts might be important for their interaction with Wls because they anchor Wnts in the membrane. Such an anchoring function might facilitate the interaction of a Wnt ligand with the integral membrane protein Wls. To address this, we used NRT-Wg, a variant of Wg, which is constitutively tethered to the membrane (Zecca et al., 1996). NRT-Wg, like Wg, induced relocalization of Wls when expressed in otherwise wild-type cells (Figure 8A,B), and lost this activity in por^{2E} cells (Figure 8B') – this despite still being

membrane-associated via the NRT-anchor. We interpret this result as an indication that the palmitoylation of Wnts by Por enables the functional interaction with Wls; endowing the Wnts with a property that goes beyond the mere association with membranes.

Discussion

Here we present a systematic analysis of all known Drosophila Wnt family members with respect to their dependence on Wls function for secretion. We found that all Wnts, with the exception of the atypical WntD, require Wls for secretion (Figure 9). This dependence correlates with the presence of a conserved Serine (in Wg: S239), which is absent in WntD (Figure 2J; S4). The Serine has been shown in mammalian Wnt3a to be acylated, suggesting that palmitoylation is in fact the key determinant. This notion is supported by our results showing that activity of the acyltransferase Porcupine is needed for the interaction of Wnts with Wls. We have also shown that the glycosylation of Wg at conserved sites does not determine its dependence on Wls. Additionally we have shown that, in apparent contrast to murine *wls*, Drosophila *wls* is not a target gene of canonical Wnt signaling.

Our conclusions on the importance of Serine acylation are supported by the recent results of Coombs et al. (2010). These authors reported that WNT3a Serine acylation is a prerequisite for its biochemical interaction with Wls. Our work is consistent with, and goes beyond, this finding by demonstrating that Serine acylation of Drosophila Wnts dictates their functional reliance on Wls for efficient secretion. It should be emphasized that our results are not proof that the Serine is acylated in all the Drosophila Wnts. Follow up experiments are needed to show that the Serine is in fact lipidated and that the acyltransferase Por is required for this function. Experiments are also needed to untangle the divergent roles and the relative importance of the Cysteine and Serine palmitoylation for secretion and signaling.

It was recently reported that overexpressed Wg^{S239A} is able to partially rescue a wing, which is mutant for *wg* (Baena-Lopez et al., 2009). This rescue was not complete as bristles were missing especially at the wing margin, where high levels of Wg signaling are needed. These results are consistent with our findings that Wg^{S239A} has some signaling activity. In our study we found that overexpressed Wg^{C93A} retained enough activity to induce the Wg target gene *sens*; whereas a Wg form carrying the S239A substitution could not (Figure S5). Interestingly in a paracrine tissue culture

assay both Wg^{C93A} and Wg^{S293A} seemed to retain some, albeit little, activity - only just above background levels. The same Wg mutants retained much higher signaling activity in an autocrine activity assay (FigS5I). The double mutant was inactive in both assays. Complicating the interpretation, Wg overexpression can partially rescue *por* mutant flies (Noordermeer et al., 1994) indicating that if the levels are high enough then even a crippled version of Wg may be able to activate the canonical signaling pathway.

A potent Wg may have to be multimerized, for example, by being inserted into and transported via lipoprotein particles. This process may necessitate the presence of the acylated Serine. The reduced ability of unacylated Wg to stimulate the pathway may also be due to its inability to access a specialized secretion mechanism. Our analysis of all Drosophila Wnts suggests that, with the exception of WntD, all Wnts utilize a similar pathway and that this requires Por and Wls. Extrapolating our and others findings it is very likely that all Wnts, including vertebrates Wnts, require Por and Wls for secretion since the Serine is completely conserved in all the species.

Acknowledgements:

We thank G. Struhl for providing Wnt cDNAs and H. Bellen for the anti-Sens antibody. We also would like to thank G. Hausmann and F. Port for discussions and critical reading of the manuscript and M. Stark for assistance with the phylogenetic tree. This work was supported by the Swiss National Science Foundation, the SystemsX.ch initiative and the European Research Council.

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Figure legends

Figure 1 Secretion of DWnts in the absence of Wls.

Expression of 3xHA tagged *hsp70*-Wnt constructs was induced by heat-shock in wing discs harboring clones homozygous mutant for *wls*^{1}. Wnt2, Wnt4, Wnt5, Wnt6 and Wnt10 accumulate in *wls* mutant cells (A-D, F), while WntD is not affected by the loss of *wls* function (E). Clone outlines are indicated in the HA stainings. Scale bar: 20µm. Analysis of the supernatant of S2R+ cells expressing pAc-Wnt-HA constructs treated with dsRNA against *wls* or *GFP* as a control by western blot. Wnt2, Wnt4, Wnt5, Wnt6 and Wnt10 are much less secreted upon *wls* dsRNA treatment (A'-D', F'). WntD is secreted to the same extent in control and *wls* dsRNA cells (E').

Figure 2 Wls relocalizes to the Golgi in cells expressing some, but not all, Wnt family members.

wls transcription is not increased in cells expressing Wg as assessed by a *wls*-lacZ reporter construct (A,B); scale bar 100µm. Expression of 3xHA tagged UAS transgenes of Wg (C), Wnt2 (D), Wnt4 (E), Wnt5 (F) Wnt6 (G) and Wnt10 (I) with *dpp*-Gal4 leads to strong Wls Golgi relocalization in the Wnt expressing cells; scale bar 20µm. No Wls Golgi relocalization is induced in cells ectopically expressing WntD (H). (J) Fragments of the alignment of all DWnts are shown to indicate the conserved Cystein 93 (asterisk) and Serine 239 (dot) of Wg.

Figure 3 Canonical signaling activity of all Wnts.

In a S2R+ co-culturing assay with untagged Wnts Wg and Wnt2 strongly induce the Wnt reporter plasmid (A). No Wnt besides Wg induces the transcription of the Wnt target gene *senseless* in vivo (B-H). Scale bar: 20µm.

Figure 4 Role of Wnt glycosylation in Wls dependent Wnt secretion.
Expression of untagged UAS transgenes of 3 single glycosylation mutant versions of Wg and the triple mutant with *dpp*-Gal4 and subsequent analysis of induction of Wls Golgi relocalization. All 4 glycosylation mutant versions of Wg still induce Wls Golgi relocalization (A-E). Untagged *hsp70*-Wg mutant constructs accumulate in wls^{1} mutant cells (A'-E'). Clone outlines are indicated in the anti-Wg stainings. Scale bar: 20µm. A Wg quadruple glycosylation mutant Wg^{N4} still requires Wls for secretion (F).

Figure 5 Wnts expressed in por^{2E} clones no longer induce Wls Golgi relocalization.
Wg and Wls co-staining in por^{2E} clones. Wg accumulates in the clone and the Wls Golgi relocalization in the Wg expressing *por* mutant cells is lost (A). 3xHA tagged Wg (B), Wnt2 (C), Wnt4 (D), Wnt5 (E), Wnt6 (F) and Wnt10 (G) expressed in por^{2E} clones using the MARCM system. All 6 Wnts can no longer induce Wls Golgi localization compared to expression in neutral control clones (B'-G'). Scale bar: 20µm.

Figure 6 Role of lipid modification of Wg in Wls dependent secretion.
Expression of untagged UAS transgenes of the Cysteine, Serine and the double mutant Wg with *dpp*-Gal4. The Cysteine mutant still induces Wls Golgi localization (B) like wild-type Wg (A) and requires Wls for secretion (B') like wild-type Wg (A'). Neither the Serine mutant Wg nor the double mutant Wg induce Wls Golgi localization (C,D), and are also secreted in wls^{1} mutant cells to the same extent as in neighbouring wild-type cells (C',D'). Clone outlines are indicated in the Wg stainings. Scale bar: 20µm.

Figure 7 Wnt2, Wnt4, Wnt5, Wnt6 and Wnt10 require the conserved Serine for Wls dependent secretion.
Expression of 3xHA tagged $Wnt2^{S202A}$ (A), $Wnt4^{S403A}$ (B), $Wnt5^{S868A}$ (C), $Wnt6^{S283A}$ (D) and $Wnt10^{S266A}$ (E) in the wing disc fail to induce Wls Golgi relocalization. Scale bar: 20µm.

Figure 8 Membrane tethered Wg requires Por to interact with Wls.
Expression of the membrane tethered HA-NRT-Wg protein induces Wls relocalization (A). Such relocalization, however, depends on Por, as it is impaired in *por* mutant cells (B, B'). Scale bar: 20µm.

Figure 9 Model of the different secretion routes of Wnts.
Wg, Wnt2, Wnt4, Wnt5, Wnt6 and Wnt10 meet Porcupine in the ER where they are lipid modified. This lipid modification is required for their recognition by and binding to Wls in the Golgi. WntD might be secreted by the normal bulk secretion route and requires neither Porcupine nor Wls.

Figure S1 Cloning of DWnt6 and DWnt10.
DWnt 6 and DWnt 10 were recloned from the original cDNAs according to the latest annotation on Flybase. We implemented data from ESTs, RNAseq and tested if the resulting constructs encoded for signal peptides (SignalP3.0 Server).

Figure S2 DWnt Homologues in different vertebrate and invertebrate species.
(A) Every DWnt has a homologue in vertebrates except DWntD. (B) Tree with all Wnt family members from human (HS), Zebrafish (DR), C. elegans (CE) and Drosophila (DM). The newly cloned DWnt 6 groups together with human Wnt6 and DWnt10 groups with human and Zebrafish Wnt10.

Figure S3 Secretion of DWnts from Kc cells.
All 3xHA tagged DWnts and their corresponding Serine mutant versions are secreted to similar levels from cultured Kc cells (A). Deglycosylation by PNGaseF treatment of all Wnts collected from Kc cell supernatant indicates that the mutation at the conserved Serine residue does not interfere with glycosylation in general. All DWnts except of WntD are glycosylated (B). PNGaseF assay of Wg^{N3} and Wg^{N4} mutants in Kc cells (C).

Figure S4 Alignment of all Drosophila Wnt proteins.
The conserved lipid modification sites are indicated in red. The Cysteine (asterisk) is conserved in all DWnts, while the Serine (dot) is not conserved in WntD. The NetNGlyc1.0 server predicted potential glycosylation sites in all DWnts are indicated in green. The signal sequence that was introduced from Wg into Wnt10 is underlined in blue.

Figure S5 Canonical Wnt signalling activity of lipid modification and glycosylation mutant Wg.
Wg^{C93A} (B) induces ectopic *senseless* expression like wild-type Wg (A). The Wg^{S239A} mutant only induces *senseless* expression close to the source of endogenous Wg but not further away (C), while the $Wg^{C93A,S239A}$ double mutant has even stronger reduced

signalling activity (D). All glycosylation mutant Wg constructs expressed in the wing disc induce ectopic transcription of the Wnt target *senseless* (E-H). Scale bar: 20µm.
In the Kc cell paracrine and autocrine activity assays only the double lipid modification mutant form of Wg shows strongly reduced activity in both assays (I). Wg^{C93A}, Wg^{S239A} and Wg^{N3} retain much higher activity in the autocrine assay. Input transfection controls are shown in (I').

Figure S6 CoIP of Wls and Por with all DWnts and their corresponding Serine mutants.
Wnt-HA constructs were transfected in Kc cells. Co-IPs were performed with antibodies against against Wls (A) or the V5 epitope (B). Binding of Wnts to Wls is strictly dependent on the Serine acylation (A). Por interacts with Wg, Wnt2, Wnt4, Wnt5 Wnt6 and Wnt10. The interaction is reduced when the Serine is exchanged to Alanine (B). The TNF family ligand Eiger does neither bind to Wls nor to Por (A,B). Graphs below the blots show realtive amounts of bound proteins normalised to the input. (C) Schematic of the $WntD^{Wg235-246}$ construct.

Figure 1 Herr and Basler

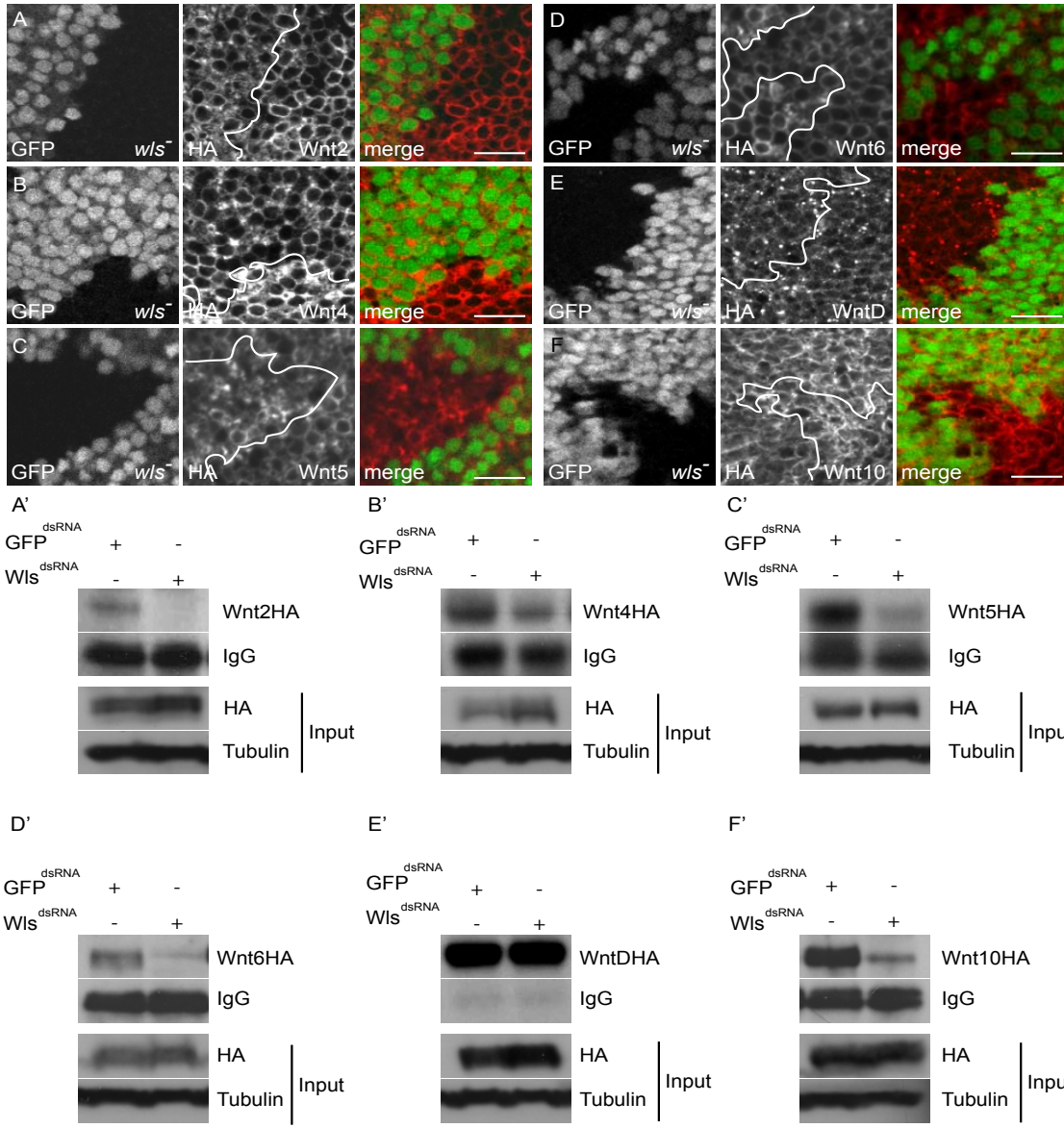
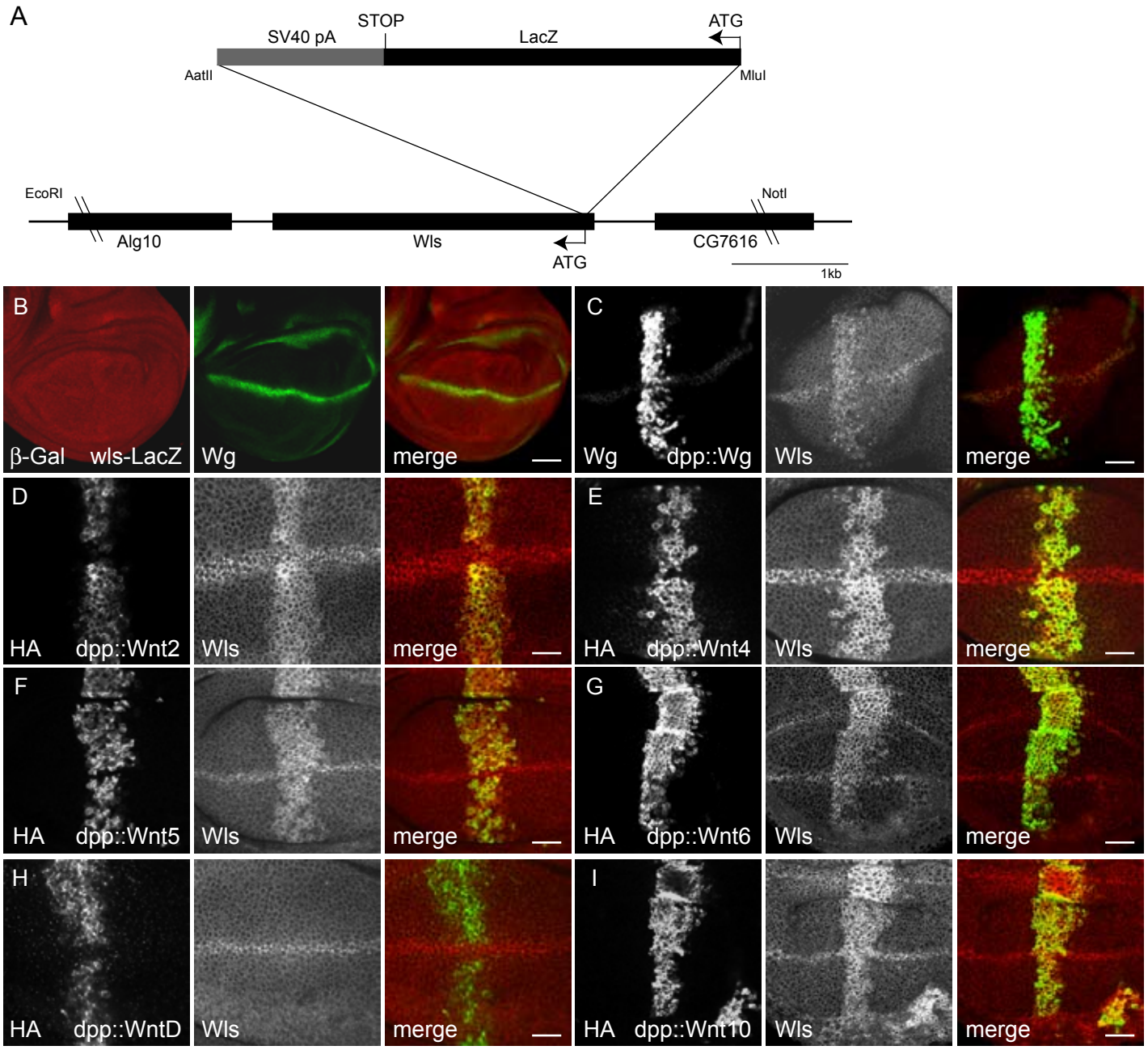


Figure 2 Herr and Basler



J

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DWnt10  77 DVTAAALEG-LDMAIREQQIQFQWHRWNCSSLST 109
DWntD   35 SWEDLTGKG-LKQALDSCQQSFQWQRWNCPSQDF  67
DWnt6   54 ALLKEIIING INLGFREQEFQFRNRRWNCTVLRK  87
DWnt5  567 SVMPAISRG-ARAAIQECQFQFKNRRWNCSTTND 599
DWnt4  257 GLPGTLSEA-RRLATTHCEEQFRYDRWNCSIETR 290
DWnt2   49 DALIALGEG-HQLGAQECQHQFRGHRWNCSEVWQ  81
Wg      77 GVLGALVKG-ANLAISECQHQFRNRRWNCSTRNF 109
                      *

DWnt10 257 MEFRCKCHG---MSGSCQLKTCWKSAPDFHIVGK 287
DWntD  162 LEQECRCKQPGAVQGECQEEECVAVLKPFEAIAQ 192
DWnt6  274 MRLECKCHG---LSGSCTVKTCWLKMPPFREVAG 304
DWnt5  858 ARITCKCHG---VSGSCSLTTCWQQLSSIREIGD 888
DWnt4  394 MMDKCKCHG---VSGSCSMKTCWKKMADFNATAT 424
DWnt2  193 LRTDCKCHG---VSGSCVMKTCWKSLPPFRLVGD 223
Wg     229 MRQECKCHG---MSGSCTVKTCWMRLANFRVIGD 259
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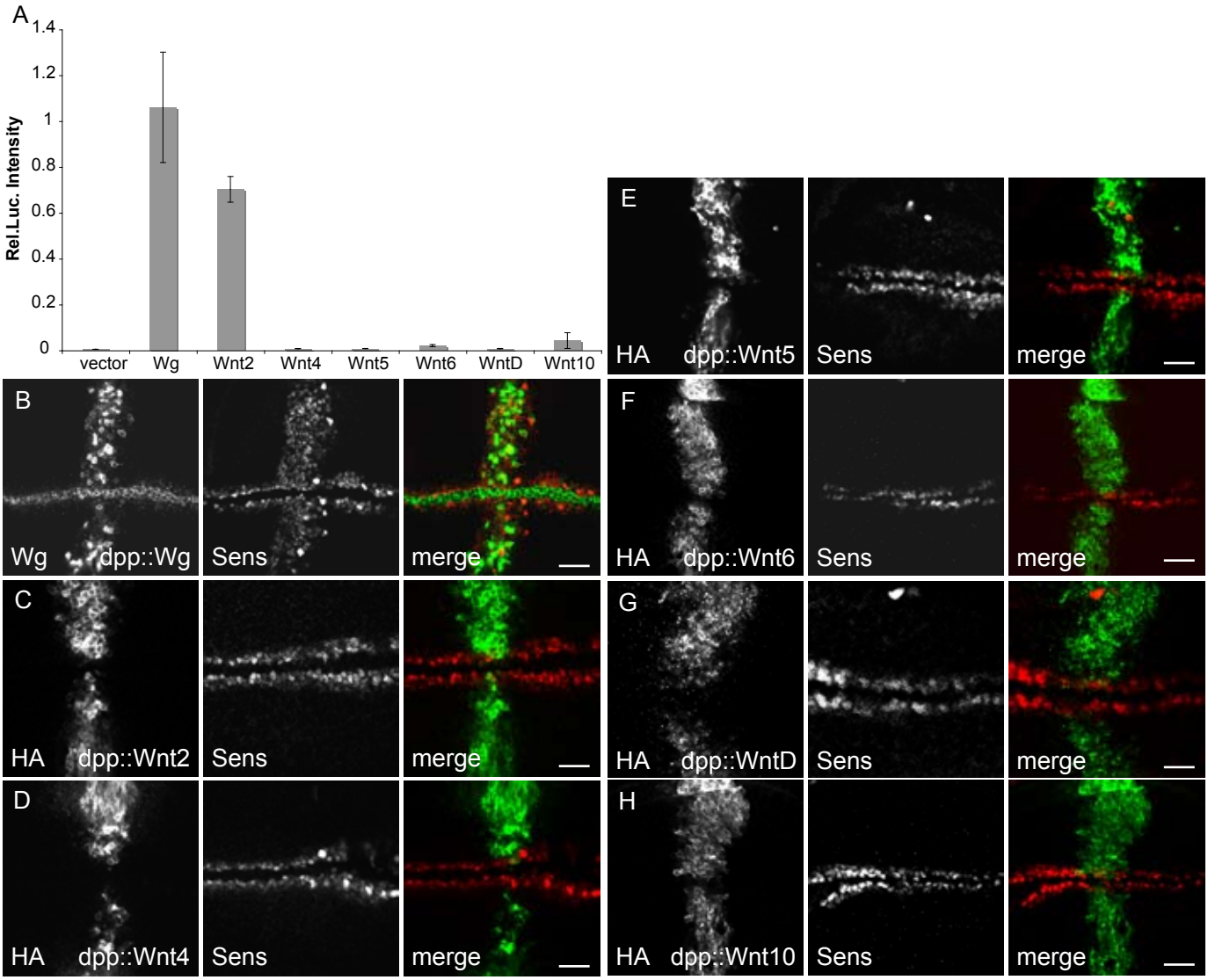
Figure 3 Herr and Basler



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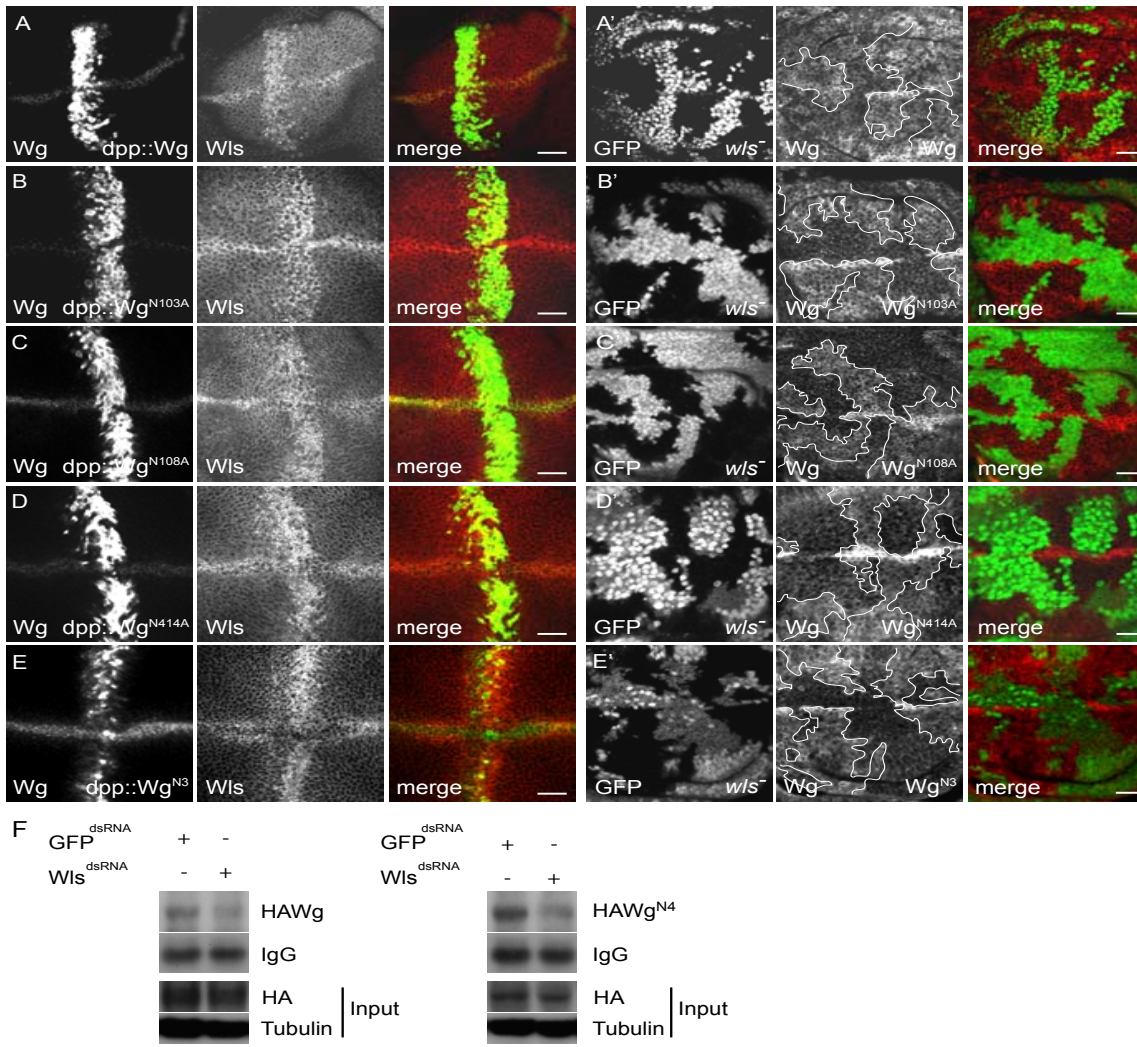

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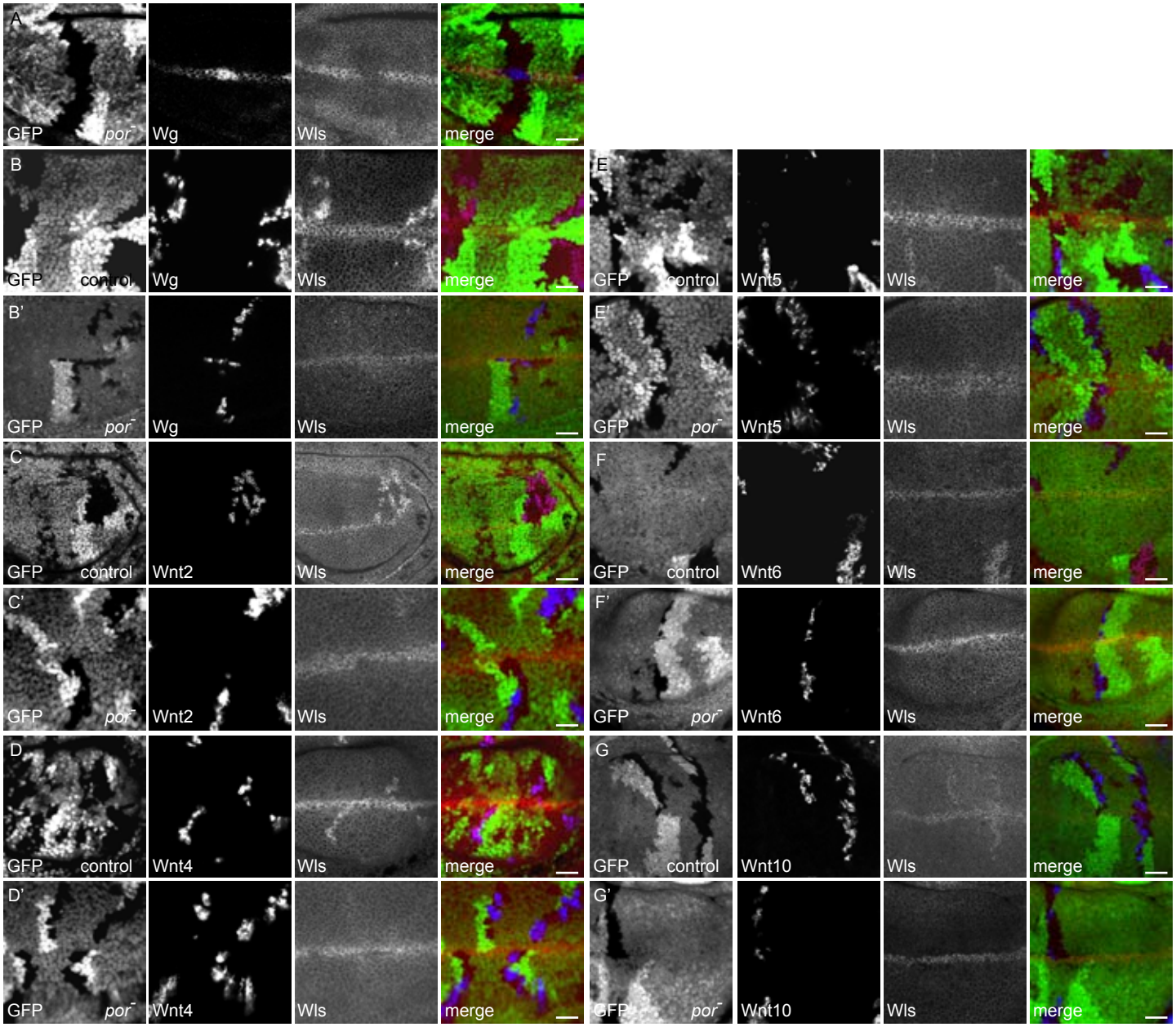

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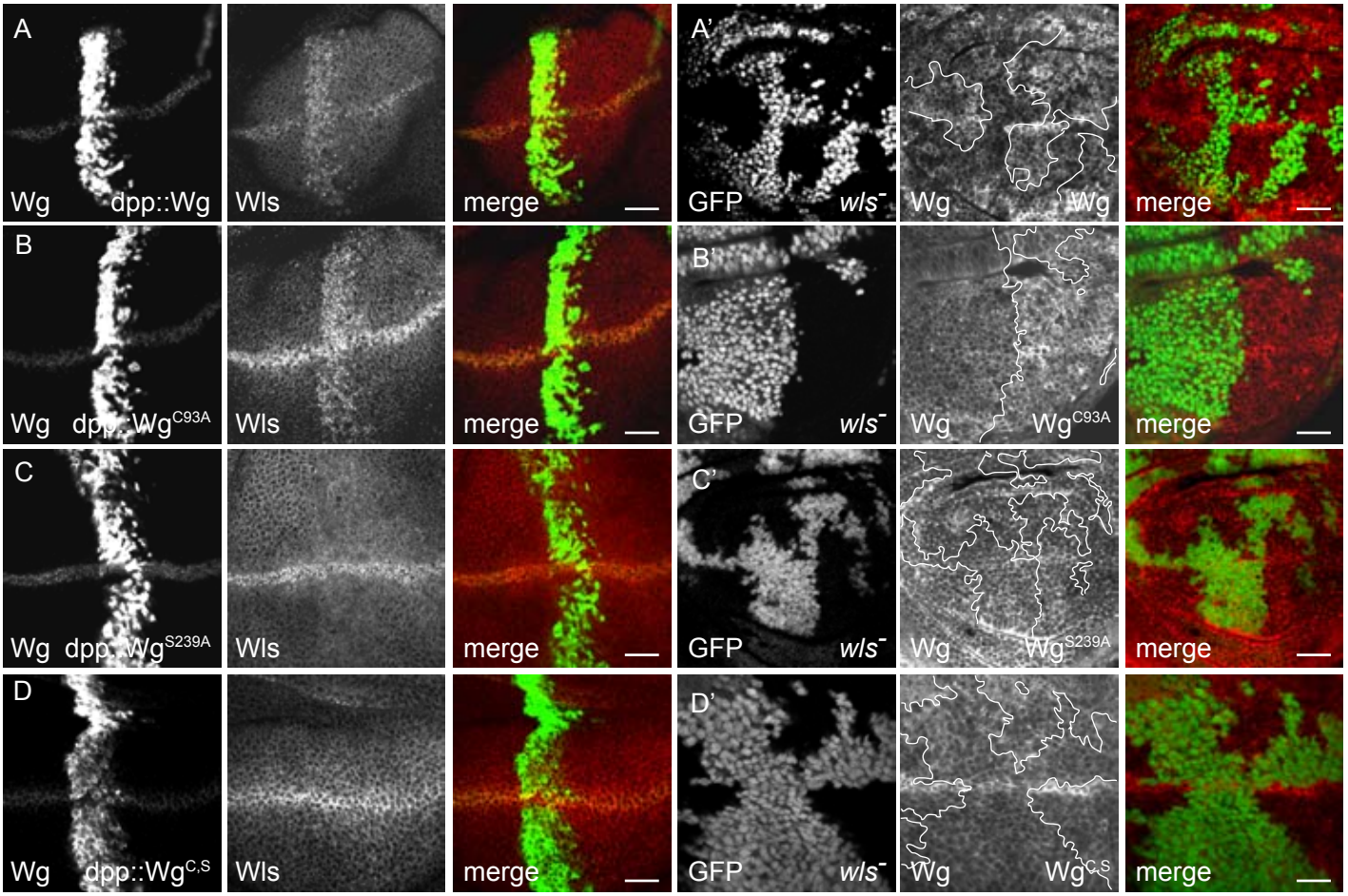

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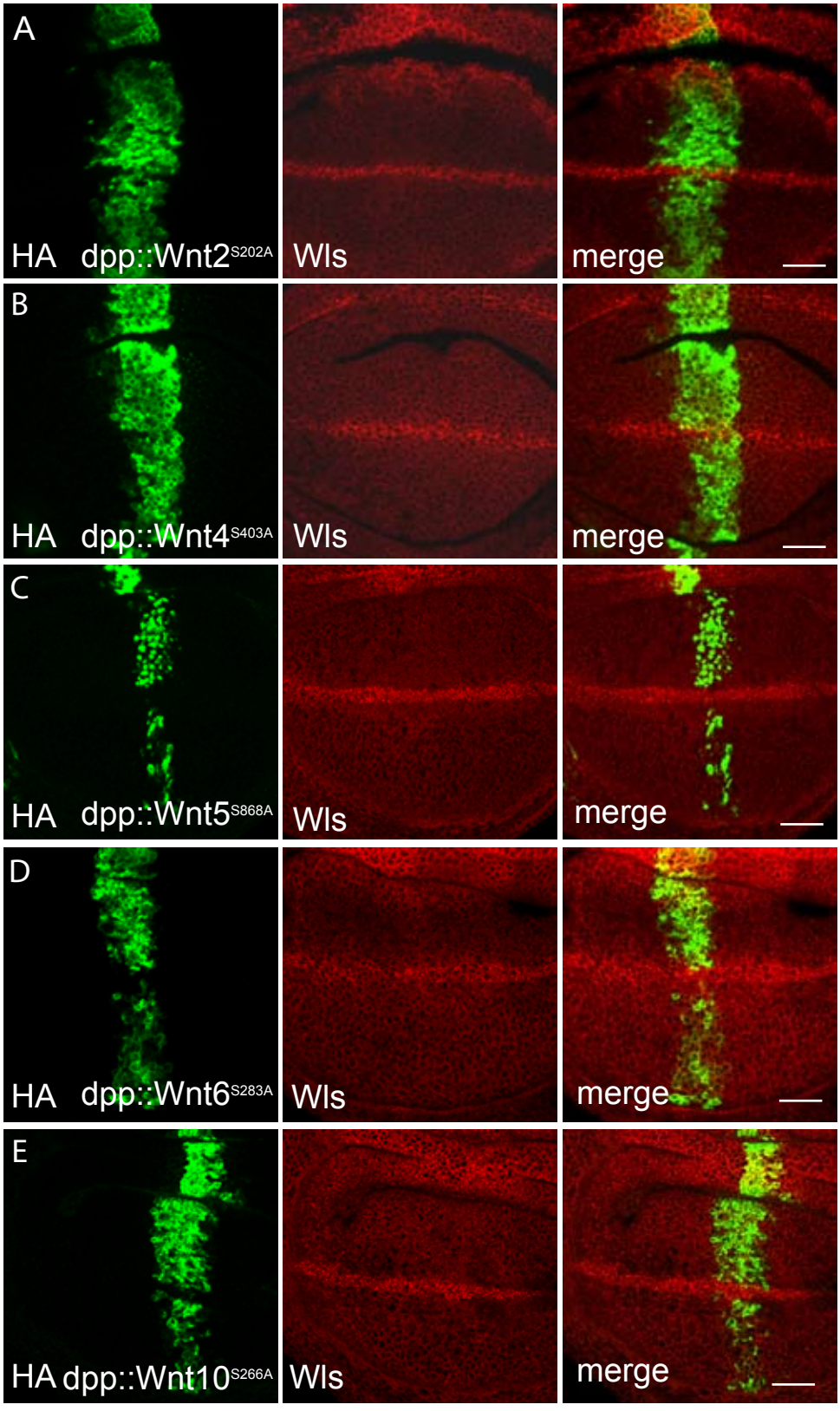

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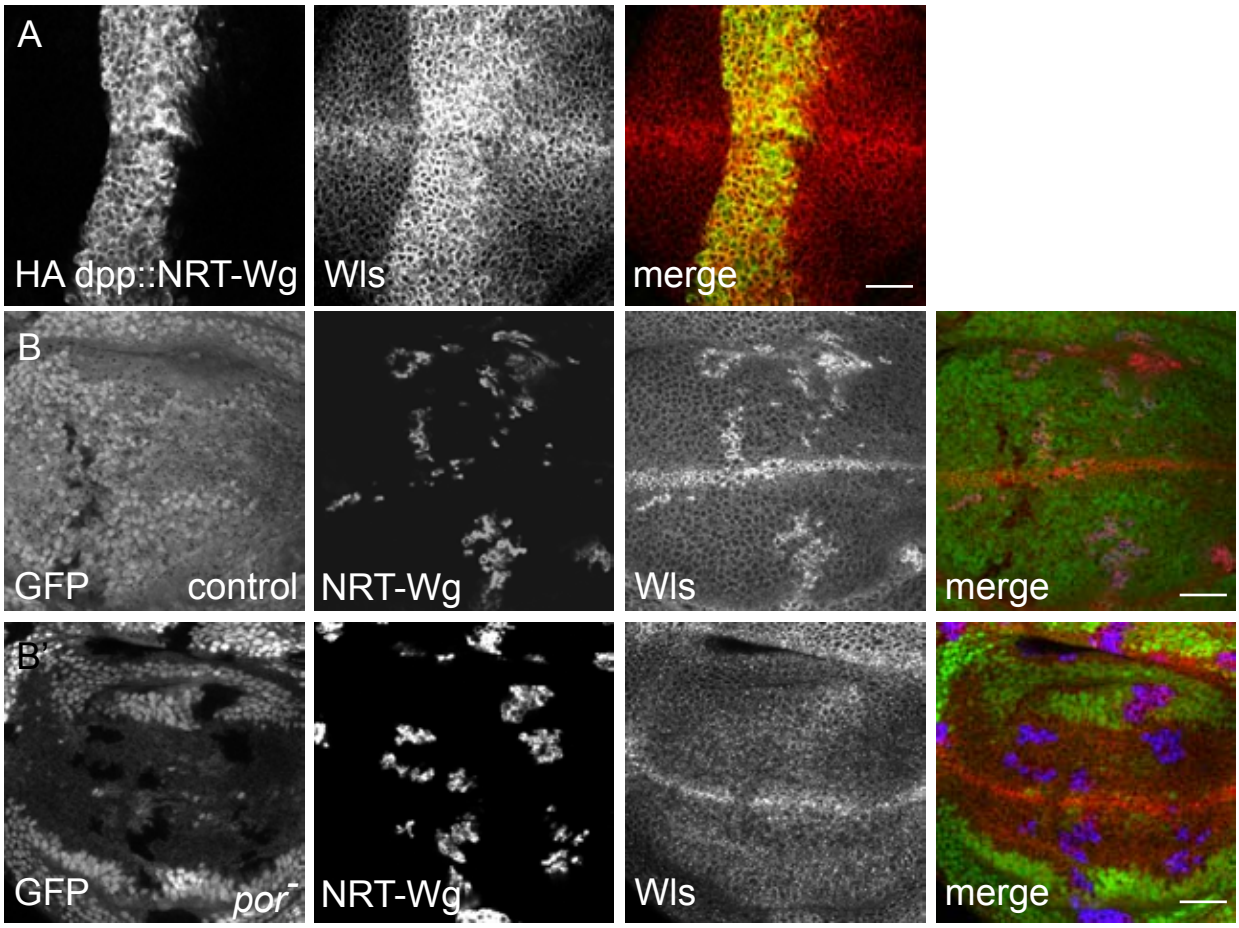


Figure 9 Herr and Basler

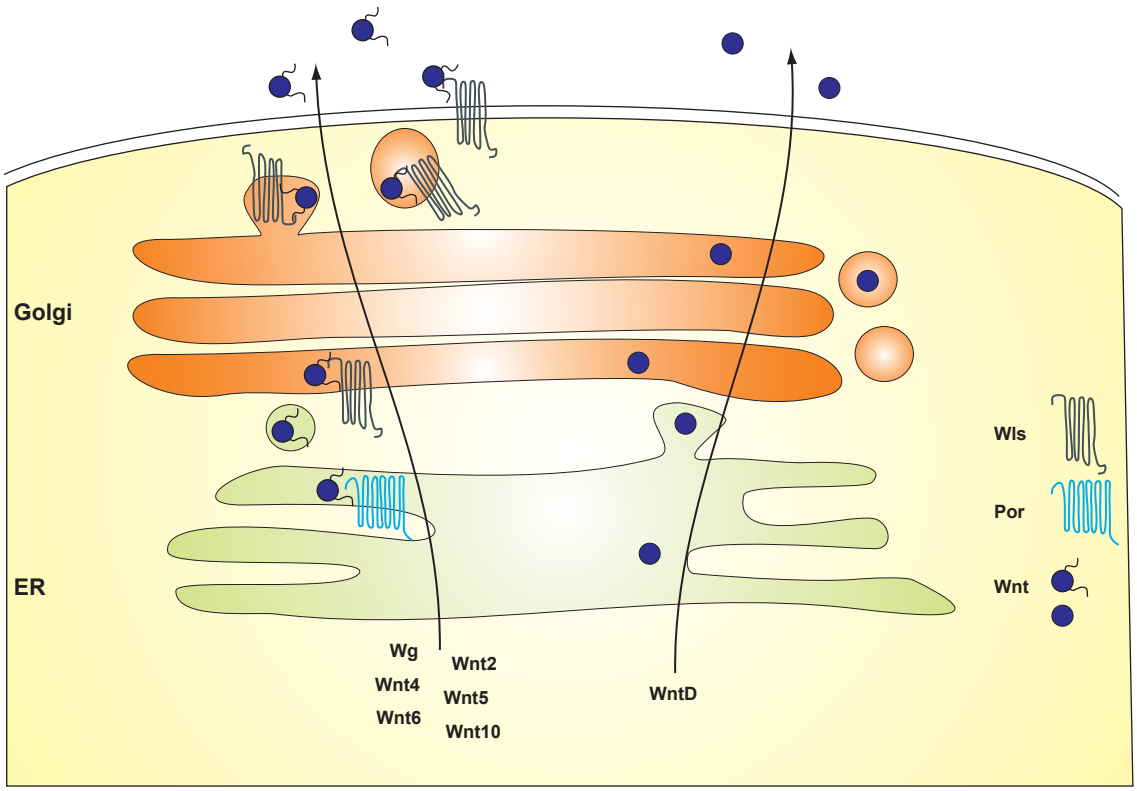
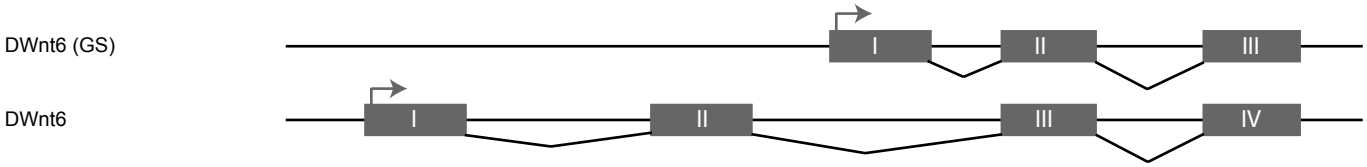
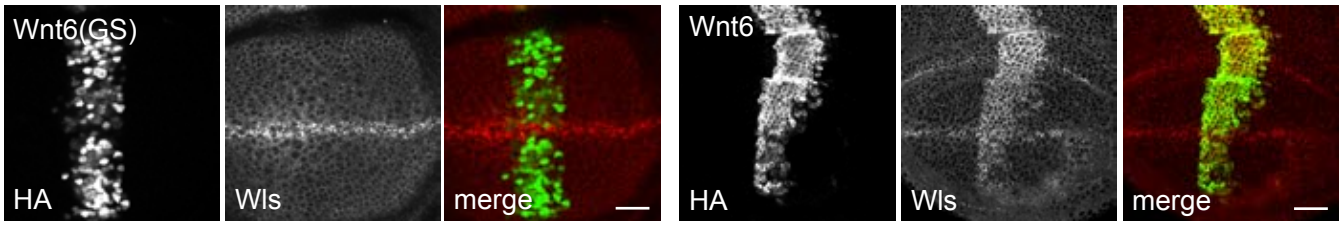
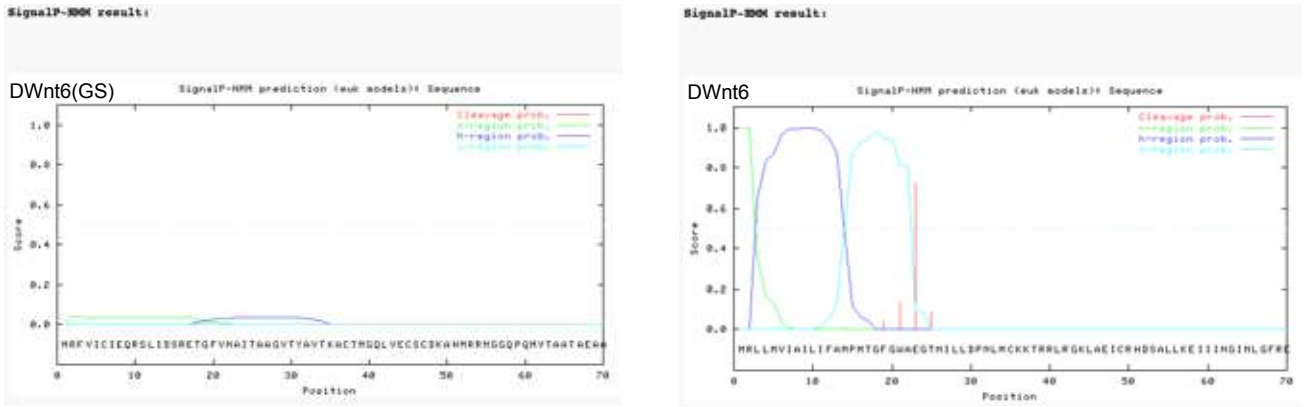


Figure S1 Herr and Basler

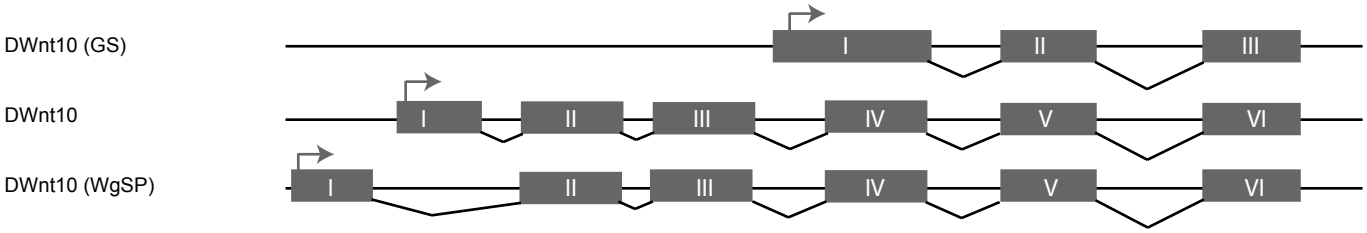
DWnt6

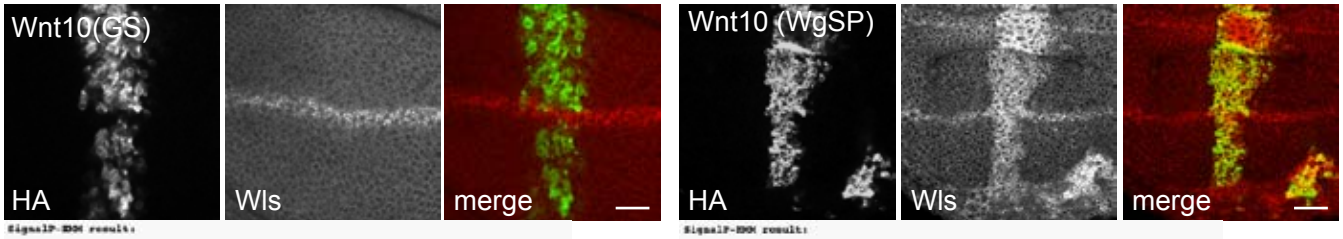






DWnt10





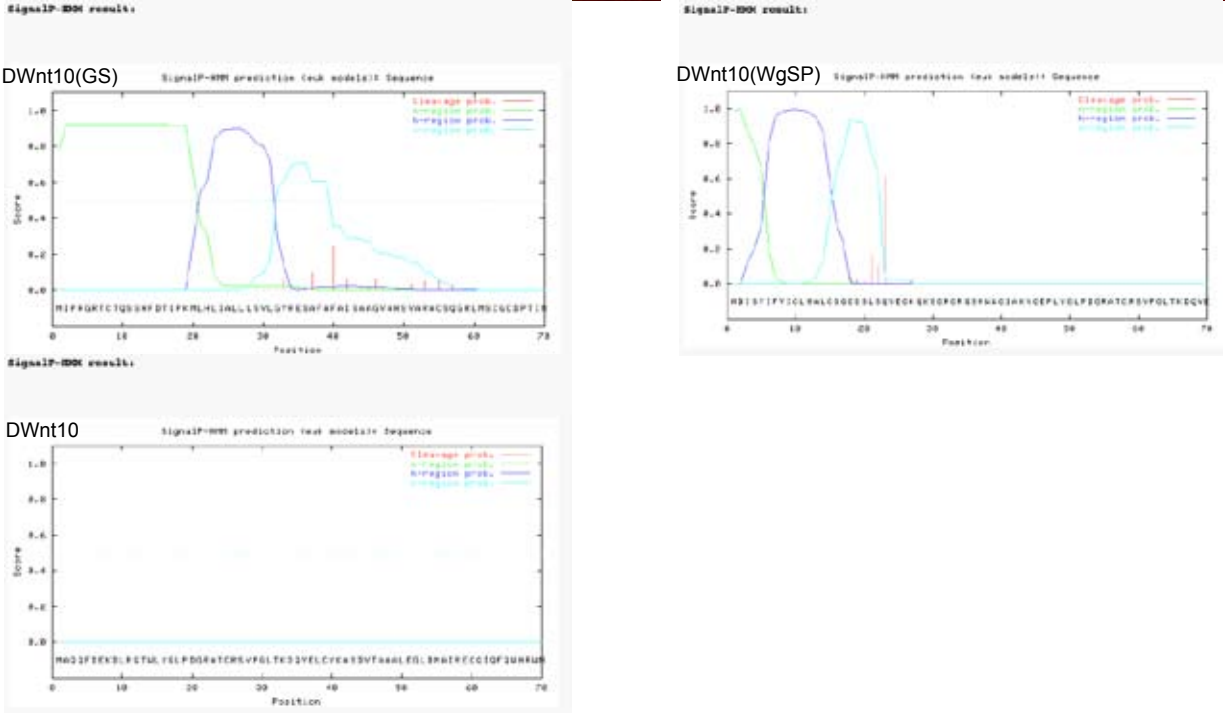


Figure S2 Herr and Basler

A

Drosophila	Wg	Wnt2	Wnt4	Wnt5	Wnt6	WntD	Wnt10
Vertebrate	Wnt1	Wnt3	Wnt9	Wnt5	Wnt6	-	Wnt10

B

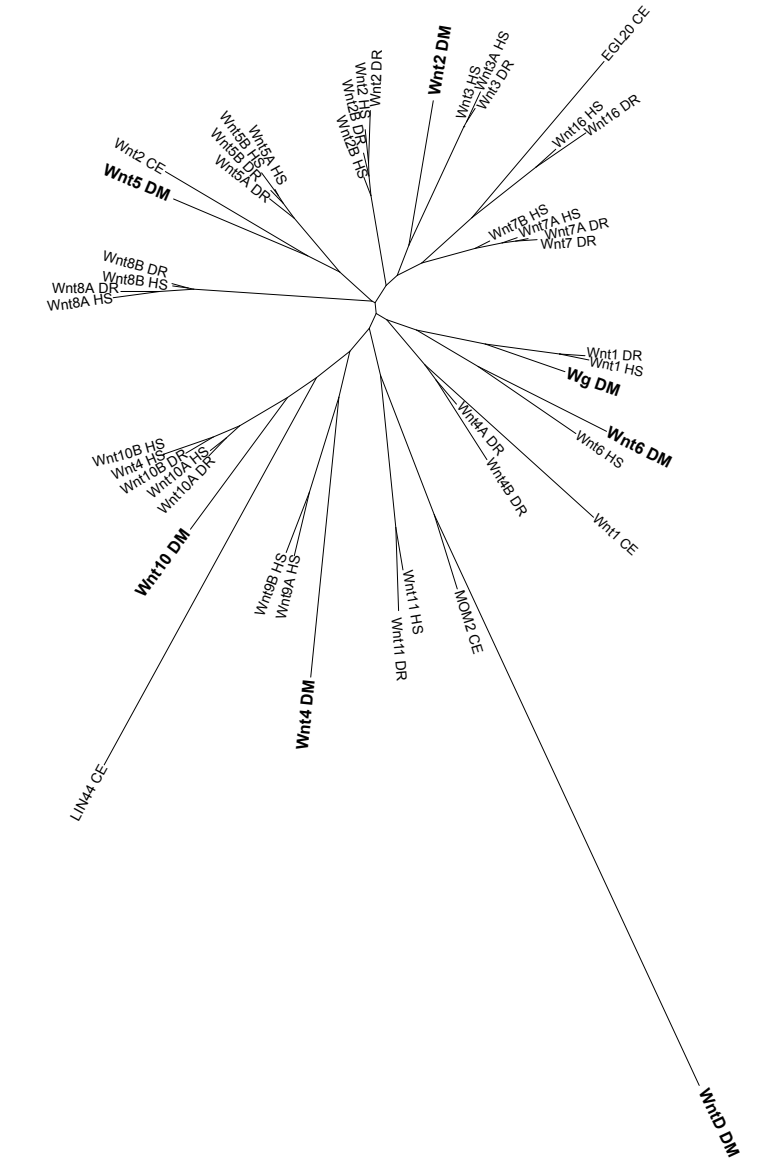


Figure S3 Herr and Basler

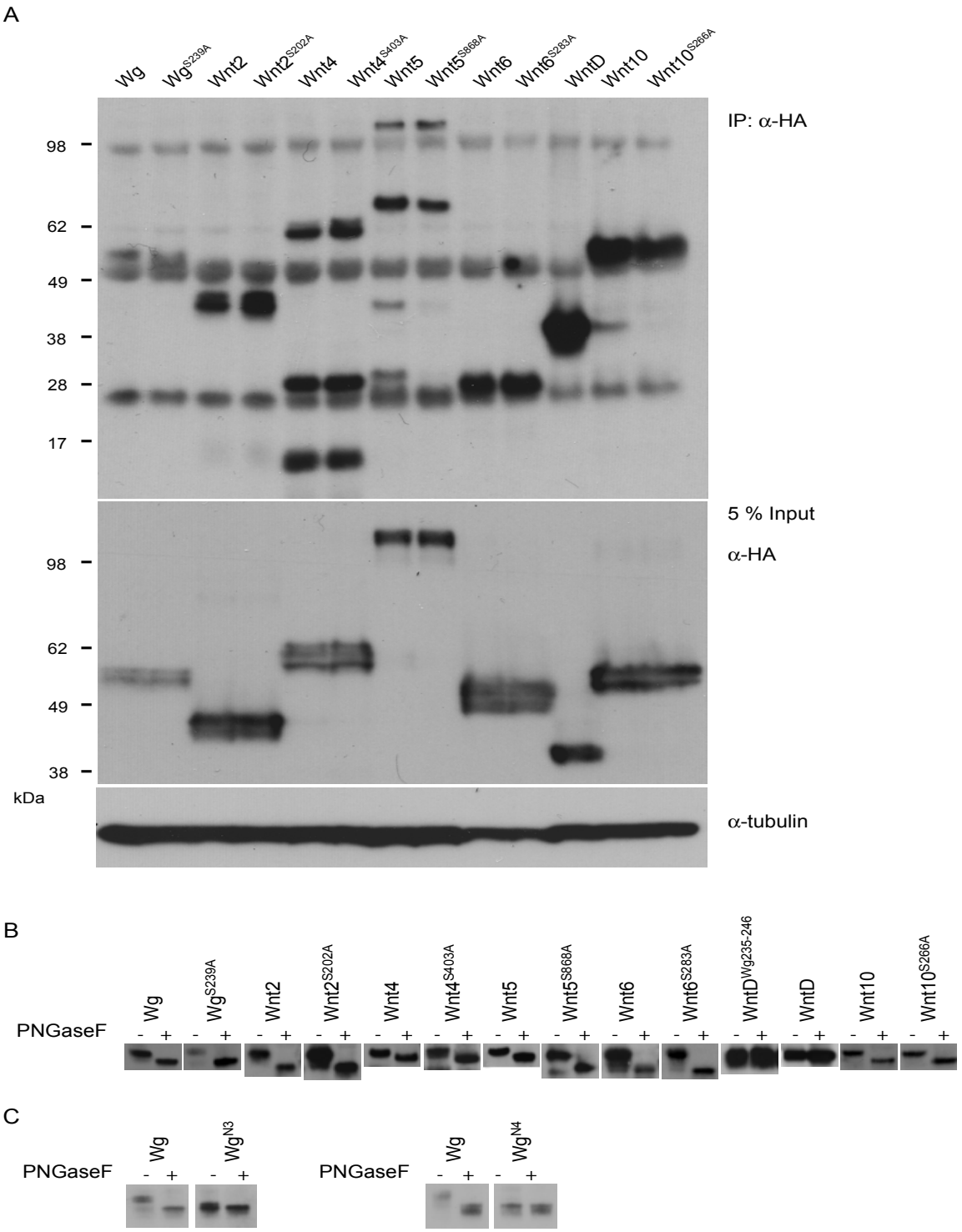


Figure S4 Herr and Basler

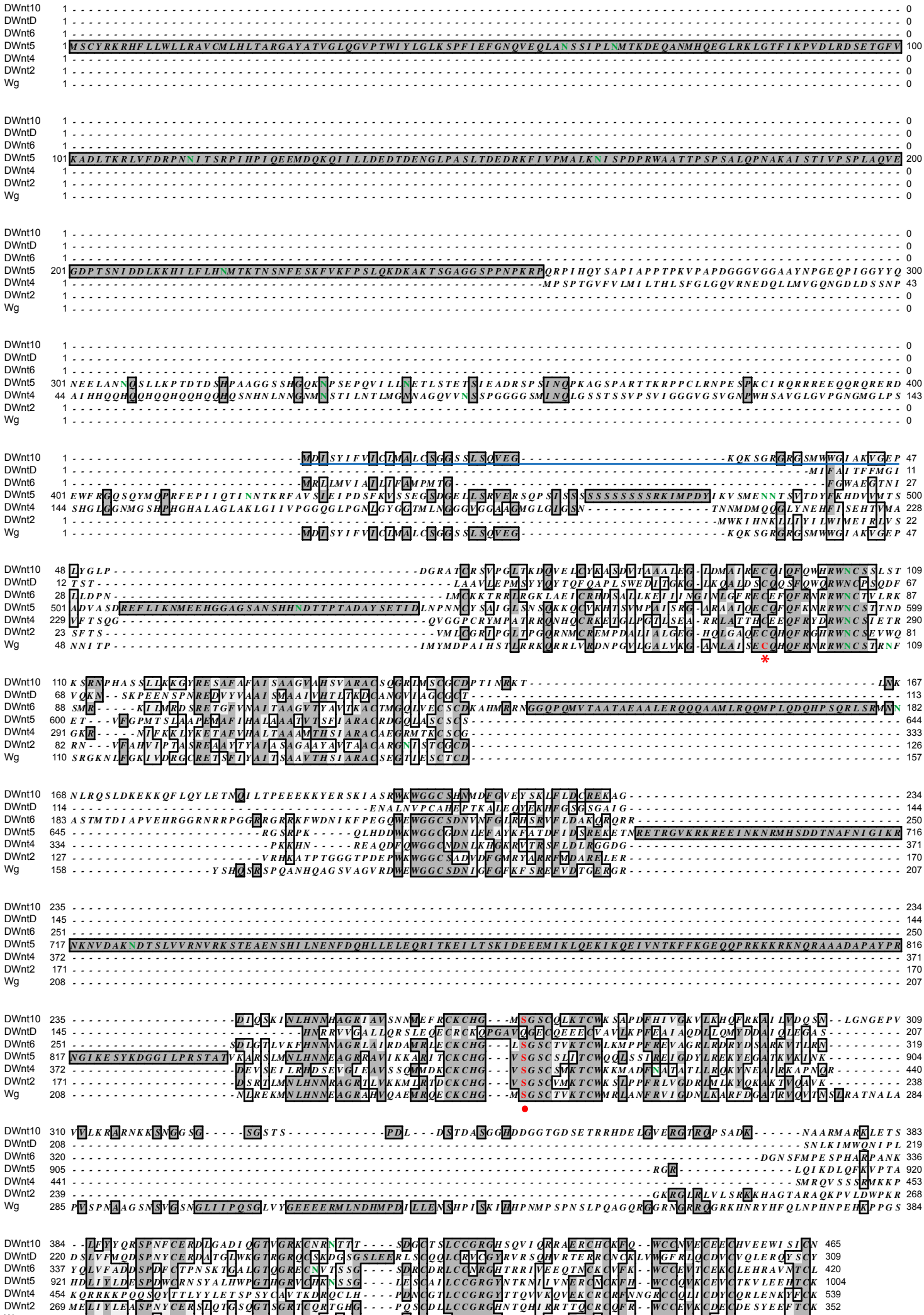
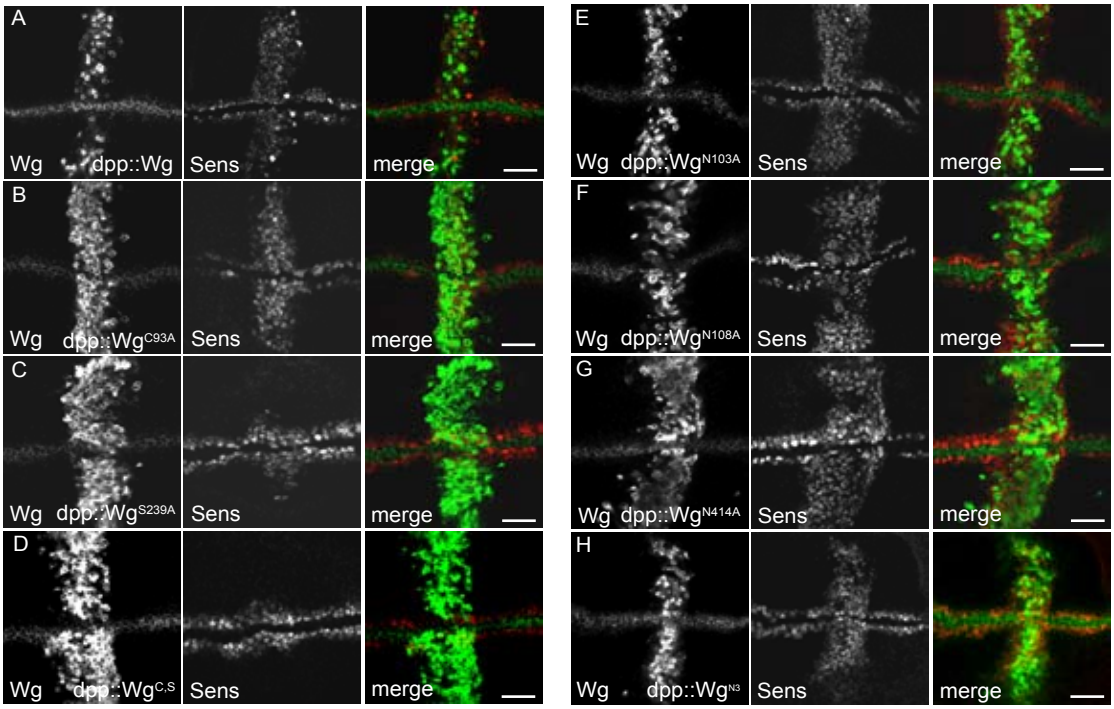
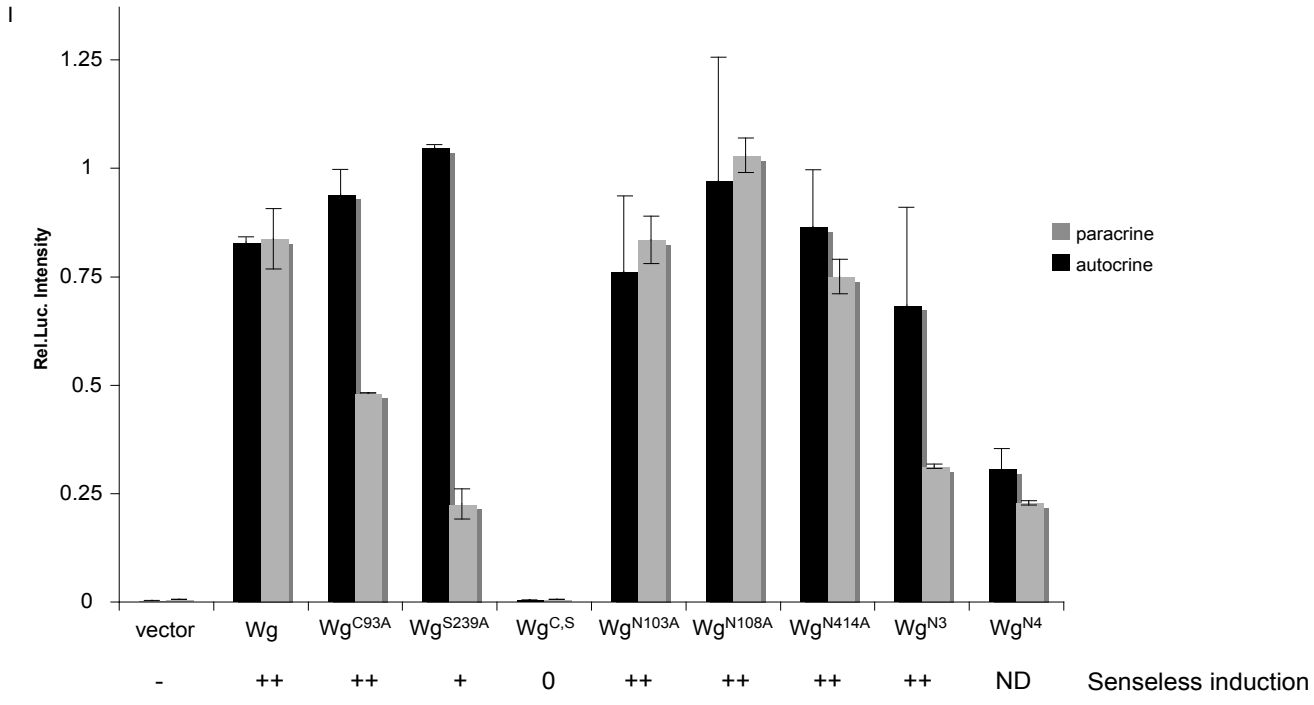


Figure S5 Herr and Basler





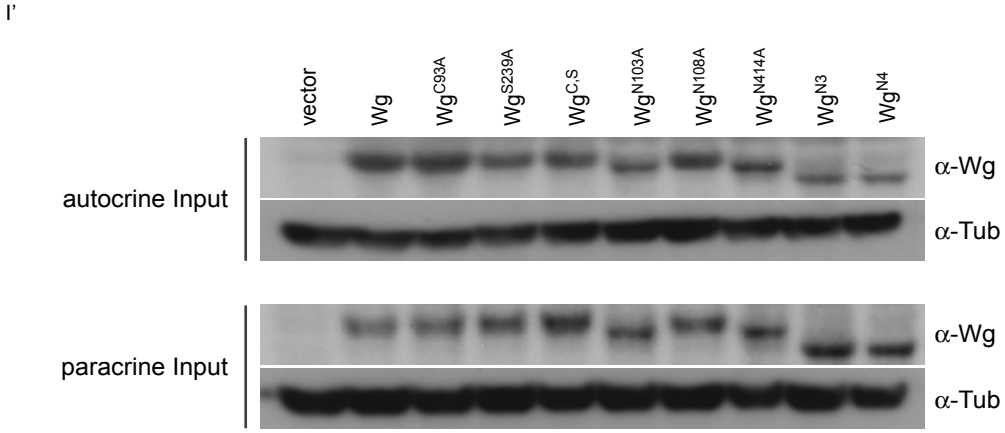
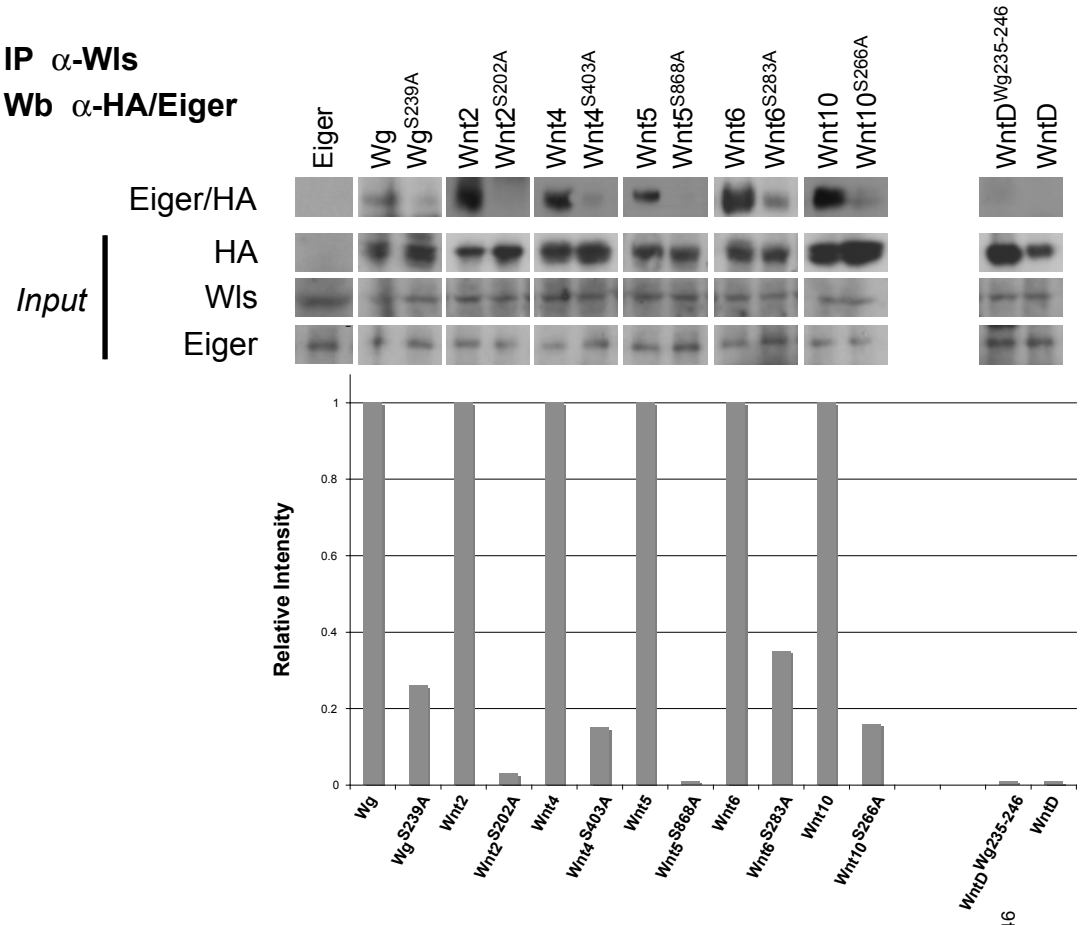
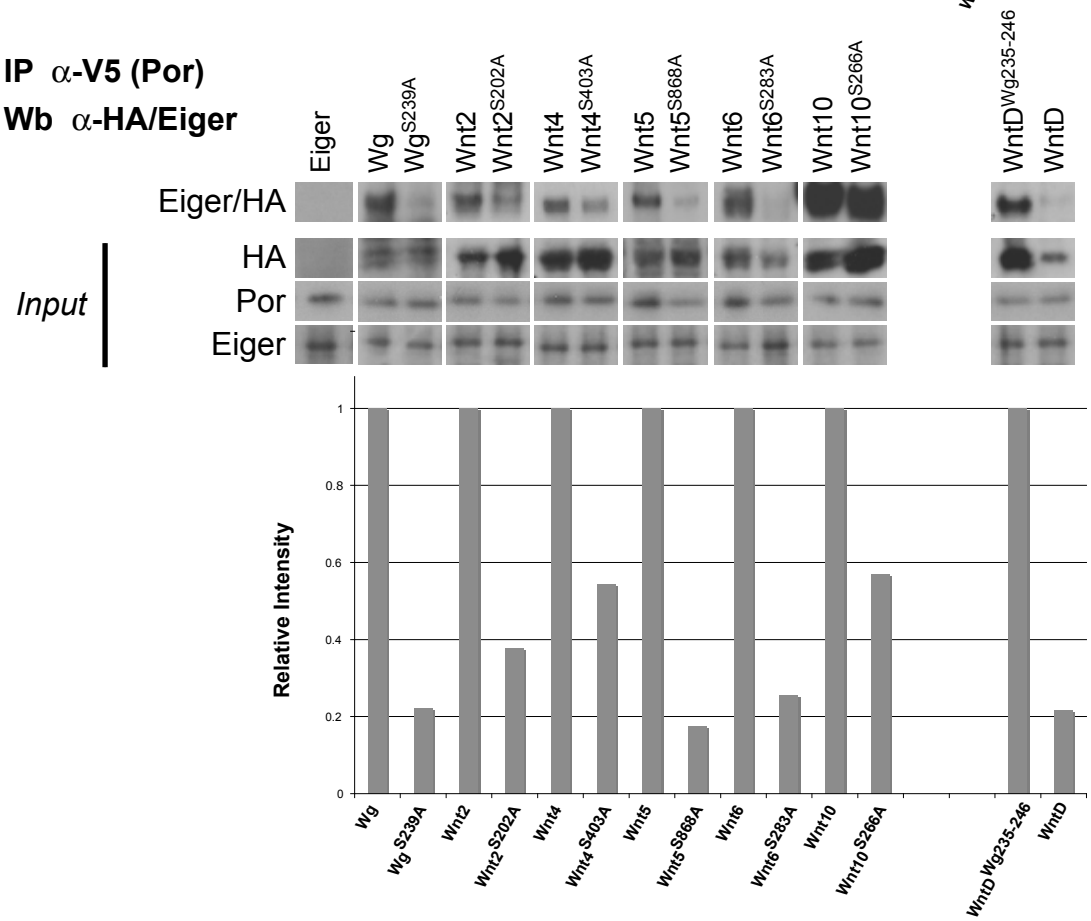


Figure S6 Herr and Basler

A



B



C